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Identification of Lhcb1/Lhcb2/Lhcb3 heterotrimers of the main light-harvesting chlorophyll *a*/*b*–protein complex of Photosystem II (LHC II)

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Abstract

Using non-denaturing isoelectric focusing in polyacrylamide vertical slab gel, we have purified to homogeneity three trimeric subcomplexes of LHC II from *Arabidopsis* thylakoid membranes. The polypeptide composition of the subcomplexes were studied by immunoblotting. Our results indicate the existence in vivo of LHC II heterotrimers containing Lhcb1, Lhcb2 and Lhcb3 gene products.

Keywords: Light harvesting complex II; Subcomplex; Trimer; (*Arabidopsis thaliana*)

1. Introduction

Light interception by PS II and the flow of excitation energy toward the reaction center is mediated by a large set of antenna pigments coordinated by specific polypeptides in the form of distinct pigment-proteins. The most abundant of them, designated LHC II, is a highly complicated system with a structural heterogeneity recognized at the levels of DNA, apoprotein and pigment–protein holocomplex. LHC II apoproteins (Lhcb1, Lhcb2 and Lhcb3) are coded for by multiple (Lhcb1) or smaller number nuclear genes (Lhcb2, Lhcb3) belonging to the LHC supergene family [1]. The Lhc proteins are synthesized in the cytoplasm as soluble precursors containing N-terminal transit peptide and post-translationally imported into chloroplasts [2]. When studied by conventional electrophoretic techniques LHC II apoproteins migrate as 2–3 bands with molecular mass between 25 and 30 kDa [3] while in high-resolution systems as many as 8–11 individual apoproteins can be identified, differing with respect to molecular mass [4] or p*I* values [5]. The heterogeneity of LHC II in vivo is considered to result from heterogeneity of coding genes [6] (*Arabidopsis* contain, e.g., five Lhcb1, four Lhcb2 and one Lhcb3 gene) rather than different pathways of cytoplasmic precursor processing, although there are also data indicating that posttranslational modifications could result in

Abbreviations: CBB G-250, Coomassie Brilliant blue G-250; CD, circular dichroism; Chl, chlorophyll; DM, *n*-dodecyl-β-dodecylmaltoside; EDTA, ethylenediaminetetraacetic acid; IEF, isoelectric focusing; LDS, lithium dedecyl sulfate; LHC II, the main light-harvesting chlorophyll *a*/*b*–protein complex of Photosystem II; nd, non-denaturing; OG, octyl-β-D-glucopyranoside; p*I*, isoelectric point; PS II, Photosystem II; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid

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electrophoretic heterogeneity [7]. In mature, green thylakoid membranes LHC II occur preferentially as trimers [8] which can associate into larger supramolecular aggregates [9]. Trimers are heterogenous themselves, adding to the heterogeneity of LHC II-individual trimeric subcomplexes can be resolved and purified by ndIEF [10,11]. The subcomplexes have various permutations of LHC II apoproteins while spectroscopic data point to virtual identity of content and organization of Chl molecules associated with individual apoproteins [10,11]. The only plant species to date for which polypeptide composition of defined LHC II trimeric subcomplexes has been solved is carnation (*Dianthus caryophylluis* L.), namely, we found a homotrimer Lhcb(1)$_3$ as well as two kinds of heterotrimers, involving Lhcb1/Lhcb2 and Lhcb1/Lhcb3 pairs [11]. In this paper we present the direct proof that *Arabidopsis thaliana* LHC II comprises – apart from two distinct Lhcb1/Lhcb2 subcomplexes – a novel heterotrimer containing all three types of LHC II apoproteins.

2. Materials and methods

2.1. Plant material

The studies were performed on wild-type *Arabidopsis* plants (*A. thaliana* cv. *Columbia*) grown on soil for 6 weeks at 23°C at a light intensity of 150 μmol quanta $m^{-2} s^{-1}$ under a light regime of 9 h light/15 h dark.

2.2. Isolation of PS II and LHC II

PS II particles were isolated according to the method of Berthold et al. [12] with the modifications described in [13], using Triton X-100/Chl ratio of 5:1, to solubilize thylakoid membranes. LHC II was isolated from PS II particles essentially as reported earlier [9]. PS II and LHC II samples were extensively washed with 2 mM EDTA (pH 7.5), suspended in 10% glycerol and stored at −20°C.

2.3. The resolution of PS II and LHC II

For separation of fractions, PS II particles were treated with 2% DM at a 13:1 detergent/Chl ratio for 25 min at 0°C. The resulting solubilization mixture was spun for 5 min at 12 300×*g* at 4°C to remove insoluble residue and the supernatant was resolved in 7% polyacrylamide ndIEF gels as described earlier [11]. For the purpose of running on a second ndIEF gel, individual LHC II subcomplexes were electroeluted form the gel after the first run, as described previously [11]. DM and glycerol were added to the eluates to yield the final concentrations of 2% and 10%, respectively, the samples were solubilized and underwent a second ndIEF applying the same focusing conditions as during the first run.

For separation of its monomeric and trimeric forms, LHC II was pelleted, adjusted to 1.2 mg Chl/ml and LDS, OG and DM were added to yield a final concentration of 0.2%, 0.4% and 0.2%, respectively. The samples were incubated on ice for 30 min and spun in 0.2–0.8 M sucrose gradients for 6.5 h as described earlier [14].

2.4. SDS–PAGE and immunoblotting

SDS–PAGE of samples electroeluted from ndIEF gels was performed in homogeneous 14% acrylamide gels using the Laemmli [15] buffer system. The gels were fixed at 12% TCA, stained overnight with 0.08% CBB G-250 by the method of Neuhoff et al. [16] and destained in 25% methanol. To perform a relative quantification of the polypeptide bands, the gels were scanned using a Shimadzu CS-9000 Flying Spot Scanner with on-board integration system. Immunoblot studies were done as outlined in [17].

2.5. In vitro dephosphorylation

PS II particles (30 μg Chl) were centrifuged, resuspended in 50 mM Tris–HCl (pH 8.5) and incubated for 20 min at 30°C in the presence of 0.1–1.0 U of alkaline phosphatase (Boeringher Mannheim). After the incubation, the samples were centrifuged, the pellet was suspended in 10% glycerol and solubilized (25 min, 0°C) in the presence of 2% DM. The solubilization mixture was spun and resolved by ndIEF.

2.6. Spectroscopic analyses

Steady-state absorption spectra were recorded at room temperature on samples electroeluted from

the gels using a Pharmacia LKB Biochrom 4060 spectrophotometer. CD spectra were recorded on a Jobin–Yvon III spectropolarimeter. The samples were either electroeluted from the gels (LHC II subcomplexes) or harvested from sucrose gradients (LHC II monomeric and trimeric forms).

2.7. Other methods

Chl *a*/*b* ratios of samples electroeluted from the gels were determined using the spectrum reconstitution method [18].

3. Results

When PS II particles from *A. thaliana* thylakoid membranes were solubilized in the presence of 2% DM and then fractionated by ndIEF, three prominent (most acidic ones) and several minor pigmented bands were resolved (Fig. 1). The prominent bands had p*I* values of 4.10, 4.05 and 4.00 and were designated A, B and C in order of decreasing p*I*. Their abundance, spectroscopic features (data not shown), Chl *a*/*b* ratios (A, 1.11:1; B, 1.11:1; C, 1.08:1) and most acidic p*I* values clearly showed that fractions A–C represent LHC II subcomplexes. The subcomplexes occurred at a ratio very close to 1:1:1 as judged by Chl distribution. The remaining bands, spanning the p*I* range of 4.25–5.00, consisted of minor Chl *a*/*b* proteins and PS II reaction center complex (data not shown), and were not subjected to further investigation.

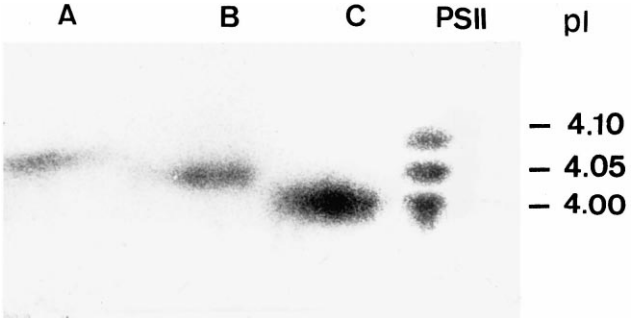

Fig. 1. Fractionation of PS II particles by ndIEF. Thirty μg Chl of PS II particles and 20 μg Chl of the LHC II subcomplexes A–C electroeluted from the gel after the preparative ndIEF was solubilized with 2% DM and resolved in a 0.75-mm-thick, analytical, 7% polyacrylamide slab gel containing 0.47% DM. In lane PS II, only a relevant portion of the gel is shown. The gel was not stained.

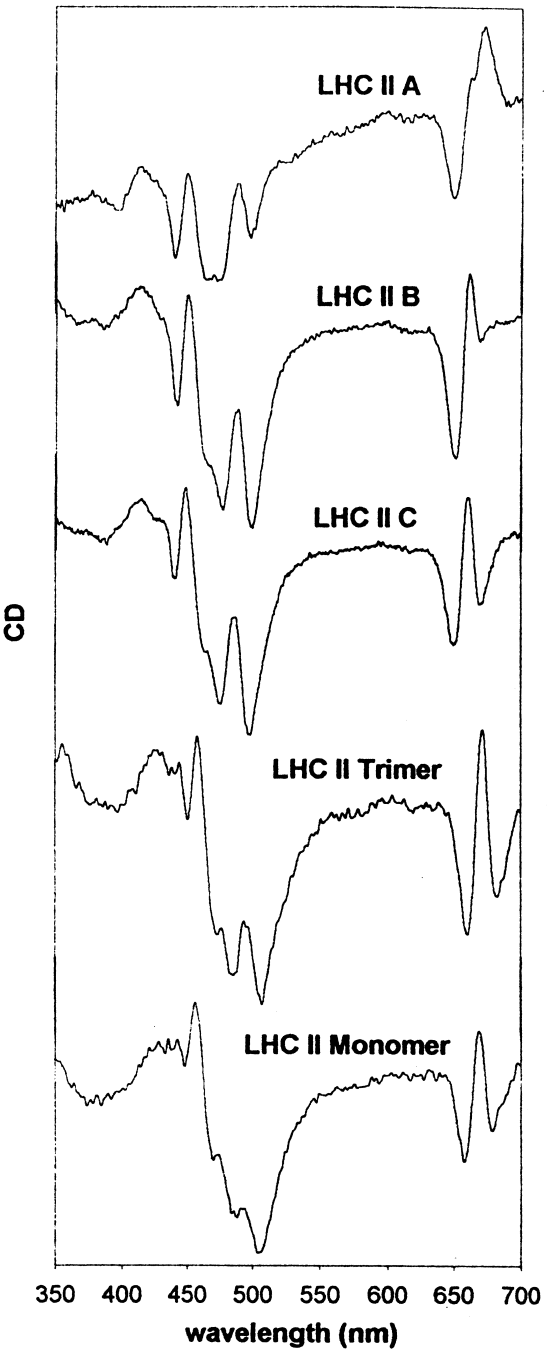

Fig. 2. Room-temperature CD spectra of LHC II subcomplexes and LHC II bulk trimers and monomers. The samples were either electroeluted from ndIEF gels (LHC II subcomplexes) or harvested from sucrose density gradients (LHC II monomers and trimers).

To assess the purity of the subcomplexes A–C, each was electroeluted from ndIEF gel, solubilized

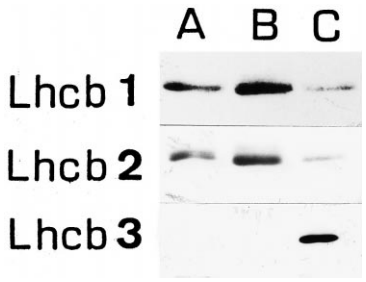

Fig. 3. Immunoblot analysis of LHC II subcomplexes. The subcomplexes were resolved by SDS–PAGE, transferred to nitrocellulose and immunostained with anti-Lhcb1, anti-Lhcb2 and anti-Lhcb3 antibodies. Chl (0.4 μg) was loaded in each lane.

in the presence of 2% DM and rerun on a second ndIEF gel. The results (Fig. 1) show that there was no sign of cross-contamination or splitting of any of the subcomplexes into closely spaced bands.

Since CD spectra of LHC II trimers and monomers are different with regard to Chl Q_y as well as Soret absorption region [19,20], we used CD spectroscopy to confirm that the subcomplexes were oligomeric. To reach this goal room-temperature CD spectra of the subcomplexes after electroelution from ndIEF gels were recorded and compared to the spectra of LHC II monomers and trimers isolated by sucrose density gradient centrifugation of bulk LHC II. Three excitonic features, which were less significant in LHC II monomer spectra, has previously been found in LHC II trimers, approximately at 648 nm (+), 478 nm (−) and 415 nm (+) [19], of which the 648 nm signal was barely noticeable at room temperature. We detected a pronounced difference between the spectra of trimeric and monomeric LHC II (Fig. 2), namely a positive excitonic feature appearing approximately at 493 nm in trimer spectra, largely reduced in case of monomers. A second, considerably less significant difference is a broad positive feature present in trimers spectra at 414 nm that is reduced in case of monomers. In spite of some minor heterogeneity, CD spectra of LHC II subcomplexes A-C were very similar to one another and to that of native LHC II trimers, i.e., all three subcomplexes clearly presented signals at 493 and 415 nm that distinguished spectra of trimers from those of monomers. This confirms that subcomplexes A–C have indeed trimeric characteristics.

In order to determine the polypeptide composition of LHC II trimeric subcomplexes in terms of *Lhcb 1–3* genes' products, the material of all subcomplexes was separated by SDS–PAGE, electrotransferred into nitrocellulose sheets and immunostained with monospecific antibodies against Lhcb1, Lhcb2 and Lhcb3. The results are shown in Fig. 3. All three subcomplexes contained a single Lhcb1 of 28.0 kDa whereas Lhcb2, although present in all subcomplexes as well, had in the A subcomplex an apparent molecular mass of 28.0 kDa and in the B and C subcomplexes apparent molecular mass of 27.5 kDa. This small but notable difference was reproducibly found in all separations. Moreover, the 28.0 kDa polypeptide migrated, equally reproducibly, as a doublet of two closely spaced bands, separated clearly on high resolution gels (Fig. 3). The Lhcb3 apoprotein had an apparent molecular mass of 29.0 kDa and was found to be present uniquely in the most acidic subcomplex (C). Thus on the basis of immunological studies it can be concluded that A and B subcomplexes are heterotimers containing Lhcb1 and Lhcb2 genes' products while subcomplex C seems to be a Lhcb1/Lhcb2/Lhcb3 heterotrimer. To analyze the stoichiometries at which Lhcb1 (28 kDa), Lhcb2 (27.5 kDa) and Lhcb3 (29 kDa) are associated in subcomplex C, we compared the CBB-staining intensity of the three polypeptides (Fig. 4). Densitometric scanning of the gel revealed that the three bands had the relative staining intensity of 1.0:0.7:0.7, respectively, compatible with a

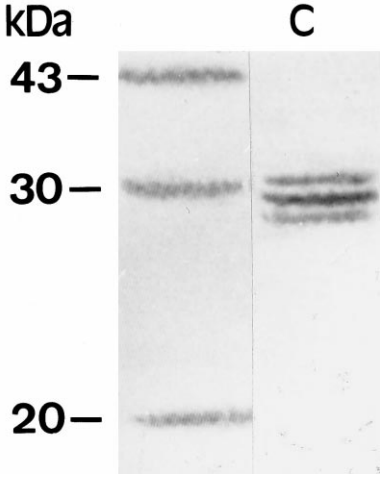

Fig. 4. Polypeptide composition of LHC II subcomplex C. 0.4 μg Chl equivalent of polypeptides of LHC II subcomplex C was resolved by SDS–PAGE and the gel was stained with CBB G-250. Apparent molecular masses of protein standards are shown on the left.

1:1:1 ratio of the proteins in the trimer, bearing in mind that the difference in individual stainability by CBB of polypeptides belonging to Lhcb family may approach 30% [21]. Lhcb1, which has the heavier staining, has more basic amino acids (arginins and lysins), which are the targets for staining, than both Lhcb 2 and Lhcb3 [21].

4. Discussion

We have purified three different LHC II trimeric subcomplexes from *A. thaliana* thylakoid membranes, labeled A–C in order of decreasing p*I* values. To confirm their oligomeric status room temperature CD spectra of the bands A–C were monitored and compared to the spectra of authentic LHC II monomers and trimers. In agreement with the spectroscopic work of Hobe et al. [20] a 414 nm positive CD signal was found by us only in our LHC II trimer spectra while the most prominent difference between *Arabidopsis* LHC II trimers and monomers involved the amplitude of the positive feature at 493 nm. This feature was described earlier for spinach LHC II [22] as 'quenching' Chl *b* exciton, gaining in strength in aggregated trimers relative to monomers. The apparent 'quenching' Chl *b* exciton was quite significant in CD spectra of *Arabidopsis* LHC II subcomplexes A–C, again confirming this way their trimeric nature.

We have found that the trimers A and B both contained Lhcb1 and Lhcb2 but that the apparent molecular mass of Lhcb2 in trimer A is larger than in trimer B. Although our data do not allow drawing direct conclusions concerning the polypeptide stoichiometry of the subcomplex, we assume they both represent Lhcb$(1)_2$ Lhcb2 heterotrimers and not Lhcb1 Lhcb$(2)_2$ ones. Two observations support this supposition. First, in the case of carnation, the only other species for which polypeptide composition of LHC II trimers was directly established [11], Lhcb$(1)_2$ Lhcb2 heterotrimers were found. Second, *Lhcb1* mRNA levels in *Arabidopsis* leaves are several times higher than those of *Lhcb2*, thus it seems highly probable that Lhcb1 is more abundant in LHC II trimers than Lhcb2. The most acidic of the trimers, C (pI = 4.00), seems to be a heterotrimer consisting of all three LHC II apoproteins (Lhcb1–3). In carnation all Lhcb3 seems to be present in Lhcb1_2 Lhcb3 heterotrimers [11] and to the best of our knowledge, this is the first evidence for the existence of Lhcb1/Lhcb2/Lhcb3 heterotrimers. The occurrence of Lhcb1/Lhcb2/Lhcb3 heterotrimers seems to be, however, a more common phenomenon as using a very similar ndIEF system we have recently been able to detect two different Lhcb1/Lhcb2/Lhcb3 subcomplexes as constituents of spinach LHC II (Pielucha and Jackowski, unpublished).

Interestingly, Lhcb3 from *Arabidopsis* thylakoid membrane were found, although smaller in size [1], to migrate as a larger polypeptide (29.0 kDa) than both Lhcb1 (28.0 kDa) and Lhcb2 (28.0+27.5 kDa). In carnation, the apparent molecular mass of Lhcb1–3 are equal (26.3 kDa) [5] while in the case of spinach (Jansson, unpublished) and in barley, the molecular mass of Lhcb3 corresponds to that of Lhcb2 one and both are only slightly smaller than Lhcb1 [23].

The fact that we recognize three different electrophoretic forms of Lhcb2 is notable. There are four Lhcb2 genes in the *Arabidopsis* genome, but three of them encode identical mature proteins and the fourth (Lhcb2.4) has only one amino acid substitution (Val to Cys). This suggests that different forms of Lhcb2 could be the result of different post-translational modifications. It is possible for example that the splitting of the 28.0 kDa band could be caused by the amino acid replacement, but there must be another element of heterogeneity involved. It is also of interest to note, that Lhcb1 and Lhcb2 in subcomplex B immunoreacted stronger with anti-Lhcb1 and anti-Lhcb2 antibodies, respectively, than Lhcb1 and Lhcb2 ones being the components of subcomplex C (Fig. 3). The CBB staining pattern of subcomplex C rules out the possibility that this complex should contain an excess of Lhcb3 polypeptides. If, as we assume, complex A and B are Lhcb1_2 Lhcb2 and C Lhcb1/Lhcb2/Lhcb3 heterotrimers, the strength of the reaction of anti-Lhcb1 polyclonal with subcomplex B should be only two times higher than in the case of C while the strength of the reaction of both subcomplexes against anti-Lhcb2 antibody should be roughly the same. We believe that the different immunoreactivity also has to do with the post-translational modification discussed above. One explanation is that subcomplex C contains phosphorylated proteins. As both relevant antibodies were raised against the domains of the polypeptides' molecules that are

close to the phosphorylation site [18], the weak reaction of the antibodies might reflect the steric hindrance imposed by the presence of the phosphate group. We do not, however, believe that this is the case since we were not able to change the ndIEF pattern by phosphatase treatment of thylakoids (data not shown). We favor instead the idea that some other, unknown post-translational modification gives rise to this heterogeneity.

To conclude, we have analyzed the polypeptide composition of three LHC II trimers prepared from *Arabidopsis* thylakoids and found evidence for a heterotrimer consisting of all three LHC II apoproteins. We also detect an unknown element of heterogeneity in the apparent molecular mass of Lhcb2 that might cause the difference in p*I* between trimers which has the same polypeptide composition.

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